IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Jeffrey A. Hubbell et al.	Art Unit:	1651
Serial No.:	09/496,231	Examiner:	A. J. Kosar
Filed:	February 1, 2000	Customer No.:	21559
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Title:	BIOMATERIALS FORMED BY NUCLEOPHILIC ADDITION REACTION TO CONJUGATED UNSATURATED GROUPS		

Commissioner for Patents
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DECLARATION OF DR. JEFFREY A. HUBBELL UNDER 37 C.F.R. з 1.132 TRAVERSING GROUNDS OF REJECTION

Under 37 C.F.R. з 1.132, I declare:

1. I am an inventor of the subject matter that is described and claimed in the above-captioned patent application.

2. My curriculum vitae is attached.

3. The invention of claim 1 is directed to a method of making a biomaterial using at least two precursors. One precursor includes at least two conjugated unsaturated bonds or groups, and another precursor includes at least two strong nucleophiles. The strong nucleophiles react with the conjugated unsaturated bonds or groups via nucleophilic addition to form the biomaterial.

4. The specification describes the self-selectivity of this reaction over sensitive biological molecules, e.g., proteins, peptides, and nucleic acids.

> The chemical reaction system of the present invention makes use of addition reactions, in which one component possesses a strong nucleophile and the other component possesses a conjugated unsaturated group, or a conjugated unsaturation. Of particular interest in this invention as strong nucleophiles are thiols. Preferably, the system makes use of conjugate addition reactions between a thiol and a conjugated unsaturation (e.g., an acrylate or a quinone). **This reaction system can be made to be self-selective, meaning substantially unreactive with other chemical groups found in most sensitive biological compounds of interest (most drugs, peptides, proteins, DNA, cells, cell aggregates, and tissues).** It is particularly useful when one or both of these components is part of a polymer or oligomer, however other possibilities are also indicated herein. (pg. 16, line 22 – pg. 17, line 6; emphasis added)

With respect to thiols and amines as nucleophiles, the specification teaches:

> Proteins contain the amino acid cysteine, the side chain of which terminates in a thiol. In spite of this, **there are very few free thiols within the protein: most proteins contain an even number of cysteine residues, and these are then paired and form disulfide cross-links between various regions of the protein. Some proteins contain an odd number of cysteine residues and most of these are present as disulfide linked dimers, again resulting in no free thiol residues being present in the native protein. Thus, there are very few free thiols in proteins.** Some important electron transferring molecules, such as glutathione, contain a free thiol, but these molecules are generally restricted in their spatial location to the inside of a cell. Accordingly, conjugated unsaturated structures presented outside the cell will be substantially unreactive with most proteins at near-physiological conditions. Amines are also nucleophiles, although not as good a nucleophile as thiols. The pH of the reaction environment is important in this consideration. In particular, unprotonated amines are generally better nucleophiles than protonated amines. **At physiological pH, amines on the side chain of lysine are almost exclusively protonated, and thus not very reactive. The alpha**

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amine of the N-terminus of peptides and proteins has a much lower pK than the side chain epsilon amine; accordingly, at physiological pH it is more reactive to conjugate additions than are the epsilon amines of the lysine side chain.

Notwithstanding, the thiol is substantially more reactive than the unprotonated amine. As stated, the pH is an important in this consideration: the deprotonated thiol is substantially more reactive than the protonated thiol. In conclusion, the addition reactions involving a conjugated unsaturation, such as an acrylate or a quinone, with a thiol, to convert two precursor components into a biomaterial will often be best carried out (meaning fastest, most self-selective) at a pH of approximately 8, where most of the thiols of interest are deprotonated (and thus more reactive) and where most of the amines of interest are still protonated (and thus less reactive). When a thiol is used as the first component, a conjugate structure that is selective in its reactivity for the thiol relative to amines is highly desirable. (pg. 17, line 7 – pg. 18, line 8; emphasis added)

5. Hubbell, Journal of Controlled Release1996, 39:305-313, of which I am the author, summarizes the results of experiments in which a hydrogel is formed via photopolymerization and used to entrap proteins (Hubbell, pp. 309 and 311). These results were previously published in West et al., Reactive Polymers 1995, 25:139-147 (hereafter "West"), of which I am also an author. As described in West, hydrogels were formed by the photopolymerization of a single precursor, a diacrylate terminated polyethylene glycol (PEG) co-polymer (West, pg. 141). The polymerization took place at pH 7.4 in HEPES buffer (West, pg. 141). The purpose of the photopolymerization experiment with proteins was to develop drug delivery vehicles (West, abstract), and the proteins included in the vehicle were not intended to become covalently bound to the

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hydrogel, as the release mechanism for the protein was diffusion, the rate of which

increased as the hydrogel degraded (West, pp. 145-146). To this effect, Hubbell stated:

> The rates of release of proteins of various sizes from a hydrogel utilizing
> PEG of molecular mass 10 000 Da are shown in Fig. 1 [35]. As expected,
> small proteins (relative to the permeability of the hydrogel, as determined
> by the molecular mass of the PEG in the gel) were released by diffusion in
> the absence of degradation, whereas larger proteins were released by
> diffusion exclusively following degradation. With this molecular mass
> PEG chain, this transition between the two regimes occurred somewhere
> between protein molecular masses of 60 000 and 150 000 Da. Within the
> regime of release independently of degradation (i.e. molecular mass of 60
> 000 Da and less), the rate of permeability of the protein through the gel was
> inversely and linearly related to the molecular mass of the protein. (pg.
> 311)

Thus, the text in Hubbell indicates clearly that protein incorporated within the PEG

diacrylate precursor and resulting PEG-based hydrogel is not covalently incorporated

within the gel and does not serve as a crosslinker within the hydrogel material.

It should further be understood that the hydrogels of Hubbell and of West are very

different than the hydrogels of the invention of claim 1. As stated in paragraph 3 above,

in the hydrogels of the invention of claim 1, one precursor includes at least two

conjugated unsaturated bonds or groups, and another precursor includes at least two

strong nucleophiles. In the hydrogels taught in Hubbell and in West, only one precursor

is present at all, that containing only unsaturated bonds. These bonds are by nature

electrophilic; however, they participate in a free radical reaction rather than an addition

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reaction. There is no strong nucleophile present; rather, only the unsaturated groups participate in the gelation reaction.

6. Lysozyme contains cysteine, lysine, serine, threonine, tyrosine, asparagine, arginine, and glutamine residues. The eight cysteine residues in lysozyme form four disulfide bonds. As such, the sulfur atoms in lysozyme are not nucleophilic, for the reasons described in paragraph 4. The disulfide bonds would have to be reduced to make the sulfur atoms nucleophilic, and this reduction would result in undesirable destabilization of the molecule. Accordingly, one skilled in the art would not employ the cysteine residues in lysozyme for covalent attachment to polymers. Moreover, disulfide-reducing conditions were not employed in West, so the lysozyme employed in West did not contain any nucleophilic thiols. The nitrogen atoms in asparagine and glutamine are not nucleophilic as they are both present in an amide group. The nitrogen atoms in arginine are also not nucleophilic as they are present in a guanidinium group. The hydroxyl groups of serine, threonine, and tyrosine are also not strongly nucleophilic, at pH 7.4 in aqueous solution. As discussed above in paragraph 4, the ε-amino group of lysine is not nucleophilic at physiological pH; the α-amino group of a protein is typically somewhat nucleophilic at pH 7.4. Thus, under the conditions employed in West, lysozyme has only a single, modestly nucleophilic group at pH 7.4, and lysozyme could not be used to cross-link with another precursor via nucleophilic addition. Further, it is clear from the results presented in West that even strongly nucleophilic groups that are

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present in proteins mixed within those materials do not participate in the reaction under the reaction conditions employed in West. In Figure 3 of West (pg. 143), the protein ovalbumin was released, as shown in the data set denoted with the numeral 4, in a manner that depended only on its molecular weight, as described in paragraph 5 above. It is noteworthy that ovalbumin possesses four unpaired cysteine residues (see, e.g., Huntington and Stein Journal of Chromatography B 2001, 756:189, pg.191). If these had participated in an addition reaction under the reaction conditions employed in West, and the ovalbumin had become covalently conjugated to the resulting hydrogel, the linear behavior observed in Figure 4 of West (page 144) would not have been observed (the fourth data point from the left). Thus, even when strong nucleophiles were present under the reaction conditions of West, coupling of protein to the electrophilic end groups on the PEG precursor did not occur.

5. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

9 September 2008
Date

Dr. Jeffrey A. Hubbell

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JEFFREY ALAN H U B B E L L, Ph.D.

Merck-Serono Chair in Drug Delivery
Professor and Director, Institute of Bioengineering
Professor, Institute of Chemical Sciences and Engineering
Ecole Polytechnique Fédérale de Lausanne (EPFL)

PERSONAL INFORMATION

Born: November 7, 1959, Kansas City, Missouri, USA
Citizenship: USA
Residency: Switzerland (Permit C; permanent resident)

University Address:
Institute of Bioengineering
Laboratory for Regenerative Medicine and
Pharmacobiology (LMRP), Station 15
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Telephone   +41 21 693 9681
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Home Address:
Chemin des Condémines 22b
CH-1028 Préverenges, Switz.
Telephone   +41 21 803 00 50
Telefax   +41 1   355 31 00
Mobile   +41 79 593 87 41

EDUCATION

Ph.D.   Chemical Engineering, Rice University, 1986
B.S.   Chemical Engineering, Kansas State University, 1982

EXPERIENCE

2004-Present   Professor. Institute of Bioengineering (Faculty of Life Sciences and Faculty of Engineering) and the Institute of Chemical Sciences and Technology (Faculty of Basic Sciences); Director, Institute of Bioengineering (http://ibi.epfl.ch)

1997-2003   Professor of Biomedical Engineering in the Department of Materials (ETH) and the Faculty of Medicine (University of Zurich); Director, Institute for Biomedical Engineering (ETH and Univ. Zurich)

1995-1997   Professor of Chemical Engineering, California Institute of Technology

1991-1994   Associate Professor of Chemical Engineering, University of Texas

1986-1991   Assistant Professor of Chemical Engineering, University of Texas

RESEARCH EMPHASIS

Our research is in the field of biomaterials and protein engineering, with applications in tissue engineering, drug delivery, and immunotherapeutics in mind. We develop novel hydrogel and nanoparticle biomaterials and novel protein therapeutics in (i) regenerative medicine, (ii) immunotherapeutics (vaccines in infectious diseases and cancer; tolerance induction in transplant medicine and autoimmunity), and (iii) delivery of small molecule and gene drugs. As molecular targets, we (i) explore molecular variants of a number of growth factors and adhesion protein morphogens, (ii) develop new release vehicles for hydrophobic immunosuppressant, anticancer and anti-proliferative small molecule drugs, and (iii) investigate new product forms of nitric oxide; further, we develop novel nonviral vectors for delivering (iv) siRNA and (v) plasmid DNA. As clinical targets (where we have reached the human clinic, so indicated; otherwise work in development), we address *novel materials* for (i) type-I diabetes (in cell transplantation to pilot clinical trials; others in immunosuppression and tolerization in development), (ii) immunosuppression, (iii) restenosis prevention, (iv) postoperative adhesion prevention, (v) cartilage protection, (vi) cancer chemotherapy, (vii) cancer immunotherapy, and (viii) infectious diseases vaccination; and *novel bioactives* for (i) bone repair (presently to Phase II clinical trials), (ii) chronic wound repair (presently to Phase II clinical trials), (iii) restenosis prevention, (iv) bladder repair, and (v) cartilage repair. Historically, we have taken a surgical sealant to the clinic and marketplace, although we no longer work in this area academically. As such, our philosophy is to target important conditions of human and animal health, to develop novel materials and bioactives to address them, and to investigate interesting biological questions with the tools that we develop along the way.

PROFESSIONAL SOCIETIES

American Association for the Advancement of Science, AAAS
American Chemical Society, ACS
American Institute for Medical and Biological Engineering, AIMBE (College of Fellows)
American Institute of Chemical Engineers, AIChE
Biomedical Engineering Society, BMES (former Board of Directors member)
Controlled Release Society, CRS
European Society for Biomaterials, ESB
International Society for Applied Cardiovascular Biology, ISACB (Executive Council)
Materials Research Society, MRS
Society for Biomaterials (President Elect, 2007/8; President, 2008/9)
Swiss Society for Biomaterials, SSB
Swiss Society for Biomedical Engineering
Tissue Engineering and Regenerative Medicine International Society (Board of Directors member)

EDITORIAL ACTIVITIES

Associate Editor, *Journal of Biomaterials Science, Polymer Edition*
Associate Editor, *Biotechnology & Bioengineering*
Executive Editor, *Advanced Drug Delivery Reviews*
Editorial Board, *Annals of Biomedical Engineering*

Editorial Board, *Acta Biomaterialia*
Editorial Board, *Biomacromolecules*
Editorial Board, *Cell Transplantation*
Editorial Board, *Journal of Controlled Release*
Editorial Board, *Journal of Polymer Science*
Editorial Board, *European Journal of Pharmaceutics and Biopharmaceutics*

AWARDS AND HONORS

2008 Alpha Chi Sigma Award, American Institute of Chemical Engineers
2008 Food, Pharmaceutical and Bioengineering Award, American Institute of Chemical Engineers
2008 Merck-Serono Chair in Drug Delivery
2007 Distinguished Alumnus Award, Rice University
2006 Gordon Conference, Biointerface Science, Co-founder and Chair
2006 George Winter Award, European Society for Biomaterials
2004 Gaden Award, John Wiley and Sons, and the American Chemical Society
2002 Körber Foundation Award for European Science
2002 Distinguished Lecturer, Institute for Biosciences and Bioengineering, Rice University
2002 Benjamin Zweifach Lecturer, University of California San Diego
2001 Skinner Lecturer, Northwestern University
2000 Elected Fellow of Biomaterials Science and Engineering
2000 Elected Fellow of the American Association for the Advancement of Science
2000 Distinguished Lecturer, Massachusetts Institute of Technology, Department of Materials Science and Engineering
1999 Britton Chance Lecturer, University of Pennsylvania
1996 Clemson Award for Applied Research, Society for Biomaterials
1996 Chair, Keystone Symposium on Tissue Engineering
1995 Elected Fellow, American Institute of Medical and Biological Engineering
1993-5 Gordon Conference, Biocompatibility of Biomaterials, Vice Chair, Chair 1995-7
1992 W.J. Kolff Award for Outstanding Research, Am. Soc. Artif. Intern. Organs
1991 American Society of Engineering Education Dow Outstanding Young Faculty Awd.
1991 Department of Chemical Engineering Teaching Award
1990 National Science Foundation Presidential Young Investigator Award
1990 College of Engineering Award for Outstanding Teaching by an Assistant Professor
1988 National Institutes of Health, First Independent Research Support and Transition Award
1988 Department of Chemical Engineering Teaching Award
1988 Dow Chemical Company Junior Faculty Award
1988 University of Texas College of Engineering, Faculty Leadership Award
1986 Outstanding Dissertation in Chemical Engineering, Rice University
1982 Atlantic Richfield Company Fellow, Rice University

MEMBERSHIP IN FEDERAL PANELS

1998 NIH Special Study Section on Tissue Engineering, Chair (USA)

1995 NAE Organizing Committee on Frontiers of Engineering (USA)
1993-7 NIH Study Section on Surgery and Bioengineering (USA)
1993 NIH Special Study Section, Division of Research Resources (USA)
1992 NIH Study Section on Surgery and Bioengineering, Ad Hoc Member (USA)
1992 NIH Study Section on Surgery, Anesthesiology and Trauma, Ad Hoc Member (USA)
1992 NIH Study Section on Bioengineering SBIR Applications, Ad Hoc Member (USA)
1991-2 NIH Working Party on Blood-Tissue-Materials Interactions (USA)
1991 NIH/NSF Study Sect. on Biomaterials-Centered Infection, Ad Hoc Member (USA)
1990 NSF Review Panel in Bioengineering and Biotechnology, Initiation Awards (USA)
1990 NSF Review Panel in Bioengineering and Biotechnology (USA)
1989 NIH Study Section on Biosensing SBIR Applications, Ad Hoc Member (USA)
1988 NSF Review Panel in Bioengineering and Research to Aid the Handicapped (USA)

MEMBERSHIP IN OTHER PANELS
2007-Present   Scientific Advisory Board, DFG Center for Regenerative Therapies Dresden
2001-2005   Scientific Advisory Board, UK Centre for Tissue Engineering
2001-   Volkswagen Foundation, Research Commission
1997-2001   Paul Scherrer Institut, Research Commission, Life Sciences Division
1996-2001   Advisor, University of Washington, Engineering Research Center on Engineered Biomaterials

THESES, DISSERTATIONS AND RESEARCH SUPERVISED AND IN PROGRESS
(Trainees having moved to professorial positions are shown in italic, along with their current university.)

Desai, Neil P.	Blood compatibility of methacrylate copolymers: An interfacial approach	MS, ChE	1988
Pohl, Phillip I.	A light scattering device for studying human blood platelet aggregation	MS, ChE	1988
Sawhney, Amarpreet	Biodegradable polymers for the prevention of postoperative adhesions	MS, ChE	1989
Desai, Neil P.	Surface modifications for enhanced blood compatibility	PhD, ChE	1991
du Laney, Tracy	Nonadhesive biomaterials with biologically enhanced albumin affinity	MS, BME	1991
Massia, Stephen P. Arizona State University	Biomaterials with enhanced cell adhesion by bioactive peptide grafting	PhD, Biology	1991
Persad, Vashti	Cell adhesive biomaterials	MS, BME	1991
Wagner, William R, University of Pittsburgh	Thrombosis on model injured blood vessels	PhD, ChE	1991
Lyckman, Alvin	Materials for nerve regeneration	Postdoc	1992

		Biology	
Pathak, Chandrashekhar	*In situ* photocuring biomedical hydrogels	Postdoc Chemistry	1992
Sawhney, Amarpreet	Enhanced biocompatibility of microencapsulation membranes for intraperitoneal islet xenografts	PhD, ChE	1992
Drumheller, Paul D.	Endothelial cell function in injury and thrombosis	PhD, ChE	1994
Hossainy, Syed A.	Cell nonadhesive biomaterials	PhD, ChE	1994
Brockmuller, Sunny	Hydrogels for drug delivery	MS, BME	1995
Frautschi, Jack	Cellulosic membranes with enhanced albumin adsorption and reduced complement activation	PhD, BME	1995
Hearn, Dianne	Bioactive materials for the promotion of cell-type selective adhesion *in vivo*	MS, ChE	1995
Herbert, Curtis B.	Biomaterials for controlled peripheral nerve regeneration	PhD, ChE	1995
Quinn, Christopher P.	Biocompatibility and stability of biosensors	PhD, ChE	1995
West, Jennifer L. Rice University	Surgical hydrogels	PhD, BME	1996
Cruise, Greg	Photopolymerization approaches for cell immunoisolation	PhD, ChE	1997
Dang, Mai Huong	Hydrogels for ocular healing	Postdoc, MSE	1997
Elbert, Donald L	Surfactants for control of biological adhesion	PhD, ChE	1997
Lindsay, Jamie	Bioactive hydrogels for arterial resurfacing	MS, ChE	1997
Noh, Insup, Seoul National University of Technology	Peptide derivatization of poly(tetrafluoroethylene) surfaces	PhD, ChE	1997
Uhlich, Thomas	Polymer hydrogels for islet xenotransplantation	Postdoc, Chem	1997
Yue, Chenyun	Hydrogels for controlled drug retention for postoperative adhesion prevention	Postdoc, Chem	1997
Kao, John, University of Wisconsin, Madison	Peptide derivatized materials for manipulating leukocyte-material interactions	Postdoc, BME	1998
Panitch, Alyssa, Arizona State University	Tissue engineering of vascular ingrowth	Postdoc, Chem	1998
Schense, Jason C.	Bioactive materials for peripheral nerve regeneration	PhD, ChE	1999
Elbert, Donald L Washington University	Environmentally responsive polymers for delivery of macromolecules to the cytoplasm	Postdoc, ChE	2000
Park, Yong Doo	Cellularly resorbable materials for MSC transplantation in cartilage repair'	Postdoc, BME	2000
Sakiyama, Shelly E,	Bioactive hydrogel biomaterials	PhD, ChE	2000

Washington University			
Schense, Jason C.	Modified fibrin gels for enhancing wound healing	PhD, ChE	2000
Schweitzer, Beat	Development of vascular grafts using modified fibrin gels	Postdoc, Biology	2000
Vernon, Brent, Arizona State University	Development of in situ gelling materials using multifunctional polymer precursors'	Postdoc, BME	2000
Winblade, Natalie D.	Self-assembling materials for steric stabilization of tissue surfaces	PhD, ChE	2000
Bearinger, Jane P.	Supermolecular adlayers of sulfide-containing macroamphiphiles	Postdoc, ChE	2001
Pratt, Alison	Cell-responsive synthetic biomaterials formed in situ	PhD, ChE	2001
Schense, Jason C.	Bioactive materials for bone repair	Postdoc, ChE	2001
Tae, Giyoong, Gwangju Institute of Science and Technology	Enzymatically triggered hydrophobic Interactions Leading to Gel Formation in Telechelic Polymers	PhD, ChE	2001
Fuchs, Rainer	Engineering a novel hepatocyte bioreactor system for application as extracorporal liner support apparatus	PhD, MSE	2002
Gössl, Andreas	Disulfide-based biomedical hydrogel gelation and degelation chemistries	Postdoc, ChE	2002
Halstenberg, Sven	Protein polymer networks for peripheral nerve regeneration	PhD, ChE	2002
Maynard, Heather D. UCLA	Peptide mimetics of heparin and heparan sulfate	Postdoc, Chemistry	2002
Metters, Andrew, Clemson University	Mathematical modeling of PEG network resorption and remodeling	Postdoc, ChE	2002
Seliktar, Dror, Technion	Functional hydrogels in vascular healing: TGF-beta- and VEGF-containing materials	Postdoc, ChE	2002
Tirelli, Nicola, University of Manchester	Novel syntheses, properties evaluation and morphogenesis for PEG-based soft matter	Group leader, Chemistry	2002
Van de Vondele, Stephane	Hepatocyte bioreactor for an extracorporeal liver support device: Bioengineering of hepatocyte phenotype via surface signaling'	PhD, MSE	2002
Cellesi, Francesco	Combined covalent and physicochemical gelation for cell encapsulation	PhD, MSE	2003
Hall, Heike	Mechanisms involved in cord and tube formation of endothelial cells	Group leader, Biology	2003
Lutolf, Matthias Ecole Polytechnique Fédérale de Lausanne	Proteolytically degradable hydrogels for cartilage and bone repair	PhD, MSE	2003

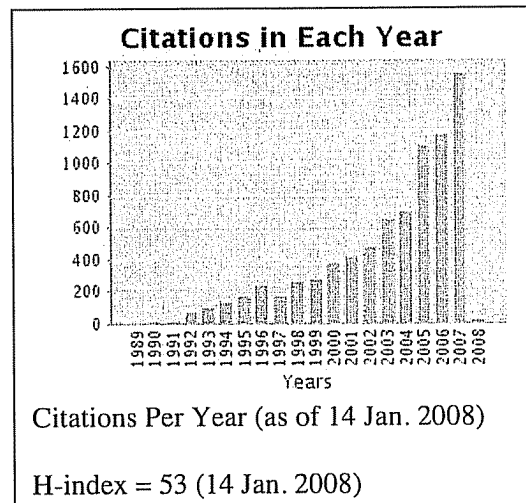| Napoli, Alessandro	Poly(propylene sulfide)-based macroamphiphiles and their self-assembly into micellar and lamellar mesophases	PhD, MSE	2003
Rizzi, Simone Queensland University of Technology	Designed protein-polymer networks for tissue engineering	PhD, MSE	2003
Valentini, Massimiliano	NMR structural characterization of polymeric vesicles and study of their interactions with proteins	Postdoc, Chemistry	2003
Zisch, Andreas	Endothelialisation signaled by protein domains presented in hydrogels	Group leader, Biol	2003
Ehrbar, Martin	VEGF variants in angiogenesis tissue engineering and angiogenesis biology	PhD, MSE	2004
Lussi, Jost	Surface patterning techniques for manipulating cell attachment at the 100 nm length scale	PhD, MSE	2004
Raeber, George	Cellular ingrowth and remodeling of PEG-based synthetic extracellular marices	PhD, MSE	2004
Rehor, Annemie	Poly(propylene sulfide) nanoparticles with application in drug delivery	PhD, MSE	2004
Thurner, Philipp	Novel staining techniques for synchretron radiation-based micro-tomography	PhD, MSE	2004
Weber-Gründler, Cornelia	Angiopoietin variant forms for angiogenesis tissue engineering	PhD, MSE	2004
Zammaretti, Prisca	Tissue engineering of angiogenesis with endothelial progenitor cells	Postdoc, ChE	2004
Cerritelli, Simona	Poly(propylene sulfide)-based polymersomes for intracellular drug targeting	Postdoc, Chemistry	2005
Feller, Lydia	Chip functionalization chemistries for bioanalytical surfaces	PhD, MSE	2005
Matthies, Annette	Biofunctional hydrogels in scar-free healing	Postdoc, Biology	2005
Missirlis, Dimitrios	Gel nanoparticles in drug delivery	PhD, MSE	2005
Trentin, Diana	Functional peptides in high-efficiency DNA delivery	PhD, MSE	2005
Van de Wetering, Petra	Functional polymers in drug delivery: the peritoneum as a target	Group Leader, Chemistry	2005
Wechsler, Sandra	PDGF variant forms for skin tissue engineering	PhD, MSE	2005
Barker, Thomas Georgia Institute of Technology	Fibrin-based biomaterials by fibrinogen protein engineering	Postdoc, BME	2006
Bermudez, Harry University of Massachusetts	Steric stabilization approaches for in vivo selection of combinatorial libraries	Postdoc, ChE	2006
Schmökel, Hugo	Orthopedic tissue engineering	Group Leader, Vet.	2006

		Med.	
Schoenmakers, Ronald	Functional polymers in drug delivery	Group Leader, Chemistry	2006
Segura, Tatiana *UCLA*	Self-assembled polymer systems for delivery of siRNA	Postdoc, ChE	2006
Adelöw, Catharina	Tissue engineering of bladder reconstruction	PhD, BioE	Current
Gengler, Malou	Cell-degradable, disulfide-based biomaterials in form of gels and colloidal particles	PhD, MSE	Current
Hasegawa, Urara	Electrostatic interactions with growth factor proteins in self-assembled complexes	Postdoc	Current
Ihm, Jong-Eun	Granulosa cell-free oocyte maturation using tissue engineering approaches	PhD, BioE	Current
Jo, Yun-Suk	Long-term NO donor biomaterials	PhE, BioE	Current
Kontos, Stephan	Expression and selection of sulfated peptides	PhD, BioE	Current
Kourtis, Iraklis	Immunofunctional nanoparticles as cancer vaccines	PhD, BioE	Current
Kraehenbuehl, Thomas	Biomimetic gel scaffolds in embryonic stem cell differentiation	PhD, BioE	Current
Lee, Seung Tae	Hydrogel systems for embryonic stem cell self-renewal and differentiation	Postdoc	Current
Lorenz, Kristen	Modulation of smooth muscle phenotype through matrix signaling	PhD, BioE	Current
Martino, Mikael	Protein engineering of fibrinogen and fibrin	PhD, BioE	Current
Michol, Lionel	Tissue engineering of the bladder wall	PhD, BioE	Current
Mochizuki, Mayumi	Matrix effects on angiogenic signaling	Postdoc, Cell Biology	Current
O'Neil, Conlin	Surface functionalization of water-dispersible macroamphiphiles	PhD, BioE	Current
Patterson, Jennifer	Therapeutic angiogenesis in myocardial repair following infarction	Postdoc	Current
Pisarek, Rubin	Polymer blends in cardiovascular devices	PhD, BioE	Current
Pullin, Brian	Tissue engineering cartilage formation with local gene therapy approaches	PhD, BioE	Current
Reddy, Sai	Lymphatic drug targeting with functionalized nanoparticles	PhD, BioE	Current
Rothenfluh, Dominique	Bioactive biomaterials as therapeutics in articular cartilage	PhD, BioE	Current
Simeoni, Eleonora	Nonviral gene delivery in bone healing	Senior sci., Molec. Biol.	Current
Van der Vlies, André	Erythrocyte-binding polymers in modulating iv pharmacodynamics	Postdoc, Chemistry	Current
Velluto, Diana	Nanoparticle formulations in drug delivery	Postdoc, Pharm. Sci.	Current
Yang, Lirong	Recombinant fibrin variants in tissue engineering	PhD, BioE	Current

COURSES TAUGHT (U, undergraduate; G, graduate)

1. CHE 102, Introduction to Chemical Engineering, undergraduate (U), Univ. Texas
2. CHE 350, Chemical Engineering Materials, (U), Univ. Texas
3. CHE 253M, Chemical Engineering Fundamentals Laboratory, (U), Univ. Texas
4. CHE 353M, Measurement, Control, and Data Analysis Laboratory, (U), Univ. Texas
5. CHE 381N, Fluid Flow and Heat Transfer, graduate (G), Univ. Texas
6. CHE 385J, Biocompatibility of Biomaterials, (G), Univ. Texas
7. 10.491, Integrated Chemical Engineering, Controlled Release Systems, (U), MIT
8. CHE 353, Transport Phenomena, (U), Univ. Texas
9. CHE 103C, Mass Transport, (U), Calif. Inst. Technol.
10. CHE 169, Biomedical Engineering - Biomaterials, (G), Calif. Inst. Technol.
11. ETH 39-620, Molecular and Cellular Aspects of Biomedical Materials, ETH and University of Zurich
12. ETH 39-718, Principals of Tissue Engineering, ETH and University of Zurich
13. ETH 39-706, Biomaterial Surfaces: Properties and Characterization, ETH Zurich
14. ETH 39-797, Materialwissenschaften
15. ETH 39-714, Biocompatible Materials I, ETH and University of Zurich
16. ETH 39-614, Biocompatible Materials II, ETH and University of Zurich
17. ETH 39-102, Biology I, ETH and University of Zurich
18. ETH 39-718, Materials for Pharmaceutical Applications, ETH and University of Zurich
19. EPFL BB-02, Materials in Tissue Engineering and Pharmaceutical Technology, EPFL
20. EPFL, Biomaterials, in the Faculty of Life Sciences and the Faculty of Basic Sciences
21. EPFL, Materials Science, in the Faculty of Life Sciences
22. EPFL, Batchelor Deign Projects in Bioengineering and Biotechnology, in the Faculty of Life Sciences

PUBLICATIONS

Citations in Each Year

Citations Per Year (as of 14 Jan. 2008)

H-index = 53 (14 Jan. 2008)

1. Velluto, D., Demurtas, D. & Hubbell, J.A. PEG-b-PPS diblock copolymer aggregates for hydrophobic drug solubilization and release: cyclosporine A as an example. *Molecular Pharmaceutics* **E-pub ahead of print** (2008).

2. Tornay, R.L., Braschler, T., Demierre, N., Steitz, B., Finka, A., Hofmann, H., Hubbell, J.A. & Renaud, P. Dielectrophoresis-based particle exchanger for the manipulation and surface functionalization of particles. *Lab on a Chip* **8**, 267-273 (2008).

3. Swartz, M.A., Hubbell, J.A. & Reddy, S.T. Lymphatic drainage function and its immunological implications: From dendritic cell homing to vaccine design. *Semin. Immunol.* **20**, 147-156 (2008).

4. Rothenfluh, D.A., Bermudez, H., O'Neil, C.P. & Hubbell, J.A. Biofunctional polymer nanoparticles for intra-articular targeting and retention in cartilage. *Nature Materials* **7**, 248-254 (2008).

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Photopolymerized hydrogel materials for drug delivery applications

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Abstract

A novel photopolymerized hydrogel material has been developed for use as a drug delivery vehicle for bioactive materials. The hydrogel precursor consists of polyethylene glycol copolymerized with an α-hydroxy acid and with acrylate termini at each end. The precursor is water-soluble and non-toxic. The precursor polymerization conditions are very mild, and polymerization can be carried out in direct contact with cells and tissues. The degradation rate and permeability of the hydrogel can be altered by changing the composition of the precursors, allowing use of this class of materials for a variety of applications. In vitro release of proteins and oligonucleotides is reported.

Keywords: Photopolymerized hydrogel; Drug delivery vehicle; Bioactive material; Water soluble; Non-toxic precursor

1. Introduction

Recent advances in biotechnology have resulted in the development of therapeutic proteins, peptides, and oligonucleotides, creating a need for suitable delivery vehicles for hydrophilic bioactive macromolecules. Matrices of hydrophobic polymers, such as ethylene-vinyl acetate copolymers and lactic acid–glycolic acid copolymers, containing powdered macromolecules, have been used for the sustained release of proteins, polysaccharides, and oligonucleotides [1], but it is sometimes difficult to homogeneously disperse hydrophilic materials within a hydrophobic polymer matrix, resulting in potentially unpredictable release profiles. Sustained systemic delivery of peptide drugs has been achieved with the use of polyethylene glycol derivatized, 'stealth' liposomes [2]. However, there still exists a need for a hydrophilic polymer which can be used for sustained release, locally or systemically, of bioactive macromolecules. In addition, the processing conditions used in the formation of the polymer matrix should be sufficiently mild to be carried out in the presence of biological materials: temperature and pH should be limited to near physiological ranges, organic solvents should be avoided, and chemical reactions which modify functional groups found on proteins should be minimized.

Our laboratory has developed a hydrogel material which may be suitable for delivery of proteins, peptides, and oligonucleotides [3]. The hydrogel is formed by photopolymerization of

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an aqueous solution of a macromolecular precursor. Bioactive materials may be dissolved in the precursor solution to ensure homogeneous dispersion throughout the hydrogel matrix after photopolymerization. The precursor consists of a central polyethylene glycol (PEG) chain with oligomeric blocks of a hydrolyzable α-hydroxy acid, or other degradable moiety, on each side. The precursor is further capped at each end with a reactive acrylate unit to allow polymerization. Photoinitiation may be used to affect polymerization at low temperatures. The majority of the precursor is comprised of PEG, thus making the precursor highly water soluble. PEG is also noted for its excellent biocompatibility, since its highly hydrated, mobile, and non-ionic character render PEG relatively resistant to protein adsorption and cell deposition. The length of the PEG segment in the precursor determines the permeability of the hydrogel as well as its physical properties. The length and composition of the α-hydroxy acid segment regulate the degradation rate of the hydrogel. Gels made with glycolide degradable links degrade in days, lactide in weeks, and ϵ-caprolactone in months in vitro [3]. Degradation occurs via hydrolysis of the ester links in the oligomeric α-hydroxy acid segment and produces PEG, an α-hydroxy acid, and oligomers of acrylic acid, all of which present a low toxicological burden [3]. This versatile design of the precursor allows the synthesis of hydrogels appropriate for a wide variety of drug delivery applications. Release periods for proteins of vastly different sizes can be varied from hours to months, depending on the degradation rate and permeability of the releasing gel.

Photopolymerized PEG hydrogels can be implanted preformed or they may be formed by photopolymerizing the macromolecular precursor in situ. The photopolymerization process does not affect cell viability, even in cell layers in direct contact with the polymer [4]. The hydrogel has been utilized previously for cell encapsulation [4]. The hydrogel is intrinsically non-adherent to cells and tissues, but if formed in direct contact with a tissue, it will adhere strongly, presumably by interdigitation with the microscopic texture of the tissue. This technique makes it possible to localize the hydrogel at a specific site, thus allowing the use of smaller doses and possibly avoiding systemic side effects. The hydrogel remains adherent to the underlying tissue throughout the degradation process [5].

One hydrogel of this type, formed from an 8000-Da PEG chain with degradable lactic acid regions, has been used as a barrier material to prevent postsurgical adhesion formation in animal models [5–7]. Adhesion formation is a frequent surgical complication which may cause pain, bowel obstruction, and infertility. An aqueous solution of the precursor and photoinitiators can be applied to tissues that have been damaged by surgical manipulation and converted to a hydrogel by exposure to long wavelength ultraviolet light to form a conformal barrier over organs and tissues at risk of adhesion formation. This technique has been found to reduce adhesion formation by 87% in a rat model [5]. It may be possible to achieve greater adhesion reduction by locally releasing fibrinolytic agents, such as tissue plasminogen activator [8], from the hydrogel barrier.

This hydrogel material has also been used to prevent thrombosis and vessel narrowing following vascular injury [9]. A thin hydrogel barrier (<20 μm) can be formed on the inner wall of blood vessels by an interfacial photopolymerization technique, wherein the photoinitiator is first adsorbed to the vessel surface and then the vessel is filled with the hydrogel precursor and exposed to light [9]. The hydrogel barrier adheres firmly to the vessel wall during degradation. In studies evaluating response to balloon injury of the carotid artery in rabbits, application of the hydrogel barrier reduced thrombosis by approximately 97% and reduced intimal thickening, the cause of vessel narrowing, by approximately 80% [9]. In both of the cases where the hydrogel has been used as a mechanical barrier, prevention of postsurgical adhesions and inhibition of thrombosis and intimal thickening, incorporation of a pharmacological agent, such as

antisense oligonucleotides [10] or antibodies to platelet-derived growth factor [11], which would be locally released from the hydrogel barrier, could potentially yield even higher efficacy.

In the current study we report the initial characterization of the in vitro release of proteins and oligonucleotides from photopolymerized PEG hydrogels. The results shown display the versatility of this class of materials and suggest that they may be applicable for a wide range of drug delivery applications.

2. Materials and methods

Precursors were synthesized and characterized as described elsewhere [3]. Briefly, dihydroxy polyethylene glycol was reacted with D,L-lactide using stannous octoate as a catalyst. This polymer was then reacted with acryloyl chloride to add an acrylate unit at each end. Specifically, the following precursors were prepared: 10,000-Da PEG with 5 lactidyl units per end (10KL5), 10,000-Da PEG with no lactidyl units (10KDA), 8000-Da PEG with 5 lactidyl units per end (8KL5), 8000-Da PEG with 3 lactidyl units per end (8KL3), 6000-Da PEG with no lactidyl units (6KDA), and 400-Da PEG with no lactidyl units (0.4KDA). All of these precursors were acrylated at both termini. Precursors without lactidyl units formed non-degradable hydrogels. Precursors were stored under argon at 0°C until use. Precursor solutions were prepared in HEPES-buffered saline (pH 7.4, 10 mM) at a concentration of 23% w/v. A 600-mg/ml solution of the photoinitiator 2,2-dimethoxy,2-phenyl acetophenone (Aldrich) was prepared in N-vinyl pyrrolidone (Aldrich), and 1.5 μl of the photoinitiator solution was added to each 1 ml of the aqueous precursor solution for a final photoinitiator concentration of 900 ppm. Various proteins or oligonucleotides were added to the precursor solutions, as described below, and the precursor solutions were poured into disk-shaped molds and converted to hydrogel form by exposure to long wavelength ultraviolet light (70 mW/cm², Black-Ray) for 20 s. The hy-

drogel discs were incubated in HEPES-buffered saline at 37°C. Protein release was evaluated using the Pierce Protein Assay (Pierce, Rockford, IL). Oligonucleotide release was evaluated using UV spectroscopy (280 nm). All studies were performed in triplicate. Release profiles were constructed based on the amount of protein or oligonucleotide, or the enzymatic activity of protein, initially incorporated in the hydrogel samples.

Swelling of the hydrogel was evaluated using 10KL5 hydrogel disks. Disks were incubated in HEPES-buffered saline at 37° C. Disks were weighed at preset time intervals, and the percentage weight gain was computed.

Release of a protein with enzymatic activity was monitored using an activity assay to verify that protein activity was maintained following photopolymerization. The release of tissue plasminogen activator (tPA, MW = 68,000 Da, Genentech, South San Francisco, CA) from a 10KL5 hydrogel was examined. tPA was added to the precursor solution at a concentration of 1 mg/ml, and each hydrogel disk consisted of 0.25 ml of the precursor and had a diameter of 1 cm. Activity of tPA released was evaluated using a chromogenic substrate (S-2288, KABI Diagnostica, Stockholm, Sweden).

A series of proteins of increasing molecular weight was incorporated into 10KL5 hydrogel disks to evaluate the dependence of the release rate on the molecular weight of the entrapped drug. Insulin (MW = 6000 Da), lysozyme (MW = 14,300 Da), lactate dehydrogenase (MW = 36,500 Da), ovalbumin (MW = 45,000 Da), bovine serum albumin (BSA, MW = 66,000 Da), and immunoglobulin G (MW = 150,000 Da) were added to aliquots of a 10KL5 precursor solution at a concentration of 1 mg/ml, and each disk consisted of 0.25 ml precursor solution and had a diameter of 1 cm. All proteins were obtained from Sigma. In addition, BSA was added to solutions of the 8KL5 and 8KL3 precursors at a concentration of 1 mg/ml using 0.25 ml per hydrogel disk. This experiment was performed to determine the effect of slightly varying the

degradation rate while leaving all other properties unchanged.

Antisense oligonucleotides are a novel class of therapeutic agents that alter disease states by interfering with transcription and translation events. The release of anti-*rev* phosphorothioate deoxyribo-oligonucleotide (5'-TCG TCG CTG TCT CCG CTT CTT CPT GCC) was evaluated using degradable (10KL5) and non-degradable (10KDA) hydrogels to determine the amount of release attributable to diffusion with and without hydrogel degradation at this precursor molecular weight. The oligonucleotide was received as a gift from Lynx Therapeutics, Inc. The oligonucleotide was added to precursor solutions at a concentration of 1 mg/ml. An amount of 0.25 ml of precursor was used for each hydrogel disk (diameter = 1 cm). In a second study, 1 mg/ml of the anti-*rev* oligonucleotide was added to the non-degradable precursor solutions 6KDA and 0.4KDA to ascertain the effect of the PEG molecular weight on the permeability of the hydrogel.

3. Results

The release of tPA from photopolymerized hydrogels was examined to determine if the pho-

topolymerization process caused loss of enzymatic activity. As shown in Fig. 1, tPA activity was highly preserved following photopolymerization and release. Release of tPA occurred over approximately a 5-day period using a 10KL5 hydrogel. The release rate over the first 24 h was somewhat greater than during later time periods. Fig. 2 shows a profile of the swelling of the hydrogel material from its initial state as polymerized from a 23% precursor solution. The period of high release rates seems to be correlated to the swelling of the hydrogel to its equilibrium state.

The release profiles of proteins varying in molecular weight from 6000 to 150,000 Da are summarized in Fig. 3. Insulin (MW = 6000 Da) was released very rapidly, being complete within approximately 2 days, while the 10KL5 hydrogel was impermeable to IgG (MW = 150,000 Da) over the time period examined. Insulin had an average release rate of approximately 47%/day, lysozyme of 41%/day, lactate dehydrogenase of 30%/day, ovalbumin of 28%/day, and bovine serum albumin of 21%/day. The average release rate was computed from the slope of the release profile from 0 to 80% release, except for IgG. Fig. 4 shows a plot of the molecular weight of the entrapped protein versus the average re-
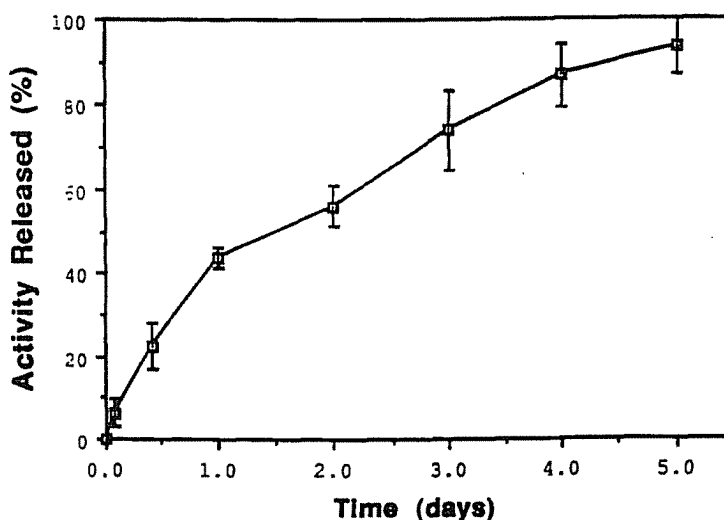

Fig. 1. Release of tPA from a degradable 10KL5 hydrogel. Activity was monitored using a chromogenic substrate assay. The photopolymerization process did not appear to reduce tPA activity. Data are means ± S.D.
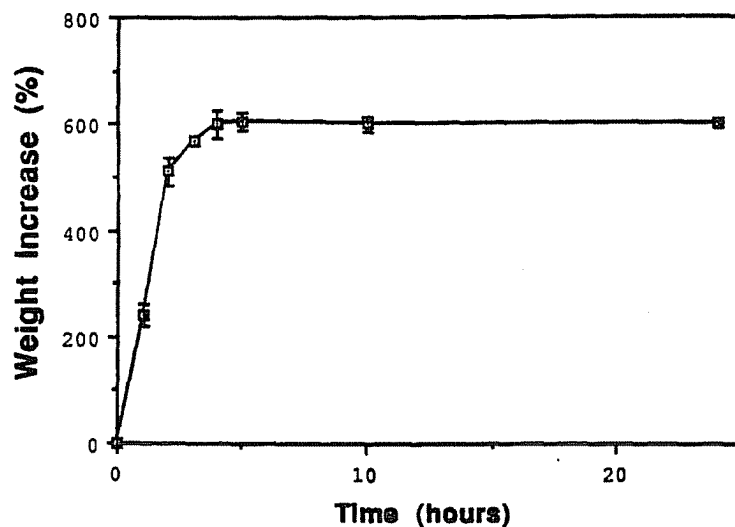
Fig. 2. Swelling of a 10KL5 hydrogel measured as the percentage weight gain over time in aqueous buffer. Data are means ± S.D.
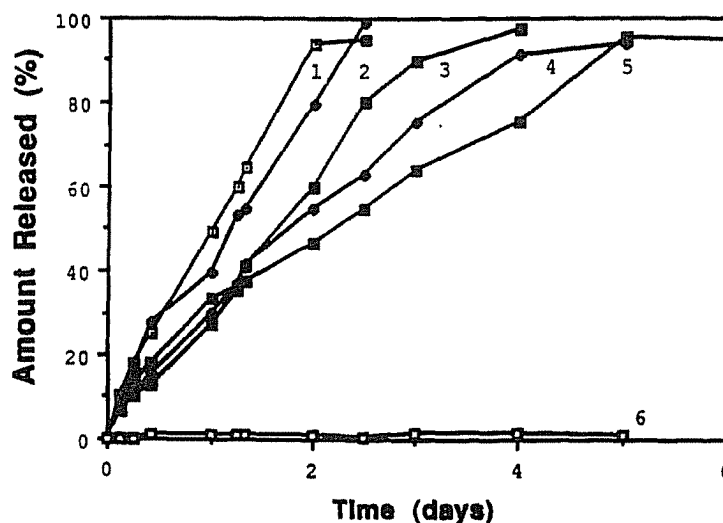

Fig. 3. A series of proteins with increasing molecular weights was released from 10KL5 hydrogels to determine the effect of the molecular weight of the permeant on the release rate. Curve 1, insulin, 6000 Da; curve 2, lysozyme, 14,300 Da; curve 3, lactate dehydrogenase, 36,500 Da; curve 4, ovalbumin, 45,000 Da; curve 5, bovine serum albumin, 66,000 Da; curve 6, immunoglobulin G, 150,000 Da.

lease rate: a linear relationship was found to exist ($R^2 = 0.98$).

BSA was released from two hydrogels with slightly different degradation rates, 8KL5 and 8KL3, as shown in Fig. 5. As expected, release from the 8KL3 hydrogel was essentially identical to release from the 8KL5 hydrogel for the first 24 h. Thereafter, release from the slower degrad-

ing 8KL3 hydrogel began lagging slightly behind release from the 8KL5 hydrogel. However, the difference in the amount released from each of the hydrogels at day 5 (when the difference was the greatest) was not significant ($P > 0.2$, Student's t-test).

Antisense oligonucleotides (anti-*rev*) were released from degradable and non-degradable hy-
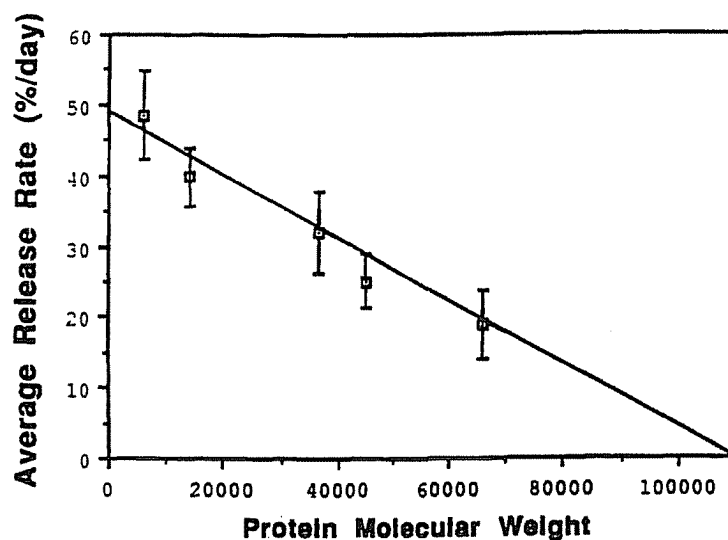
Fig. 4. The average release rates from the profiles in Fig. 3 were plotted against the molecular weights of the proteins. Data are means ± S.D.
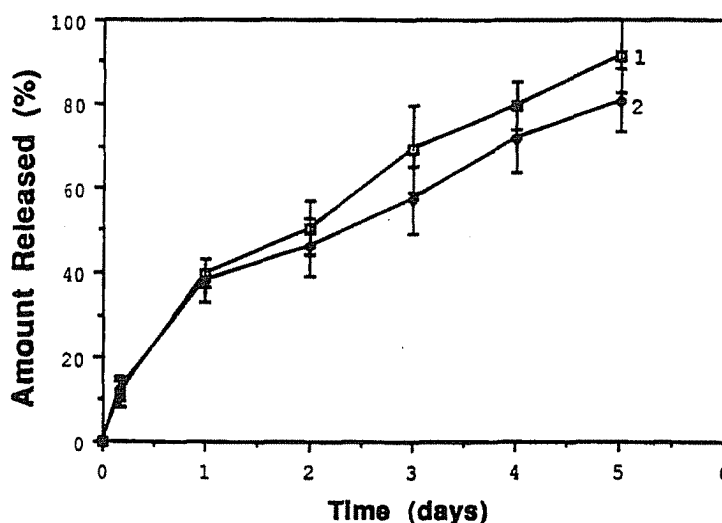

Fig. 5. Release of BSA from 8KL5 (curve 1) and 8KL3 (curve 2) hydrogels. These hydrogels have essentially the same permeability, but slightly different degradation rates. However, the difference in release rate observed was not statistically significant ($P > 0.2$).

drogels, 10KL5 and 10KDA, respectively, to determine the degree of release that resulted from diffusion of the oligonucleotide out of the matrix in the absence of degradation as compared to the increased diffusion due to hydrogel degradation. As shown in Fig. 6, release over the first 24 h appears to be largely due to diffusion, while after 24 h, very little release was seen from the non-degradable hydrogel. Release continued in the degradable gel as degradation augmented diffusion.

Fig. 7 shows the dependence of hydrogel permeability, altered by changing the molecular weight of the PEG-segment within the precursor, on the diffusional release. Non-degradable hydrogels were used so that diffusion could be
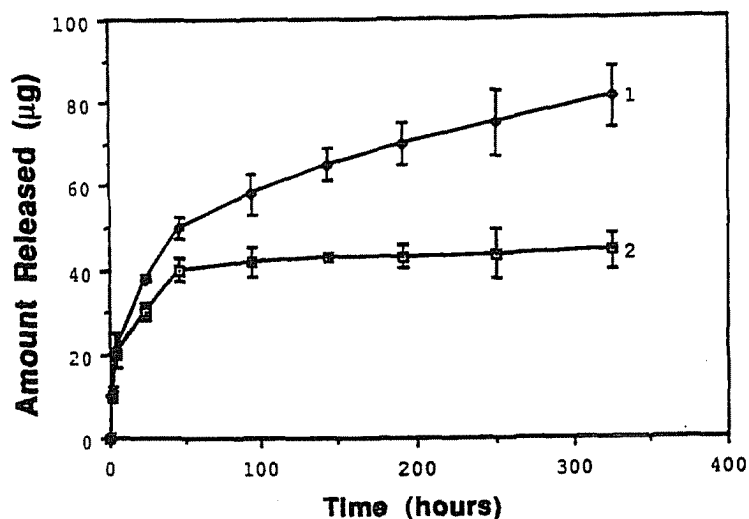
Fig. 6. Release of anti-*rev* mRNA from degradable 10KL5 (curve 1) and non-degradable 10KDA (curve 2) hydrogels. The majority of the release from 10KL5 is due to degradation of the hydrogel. Data are means ± S.D.
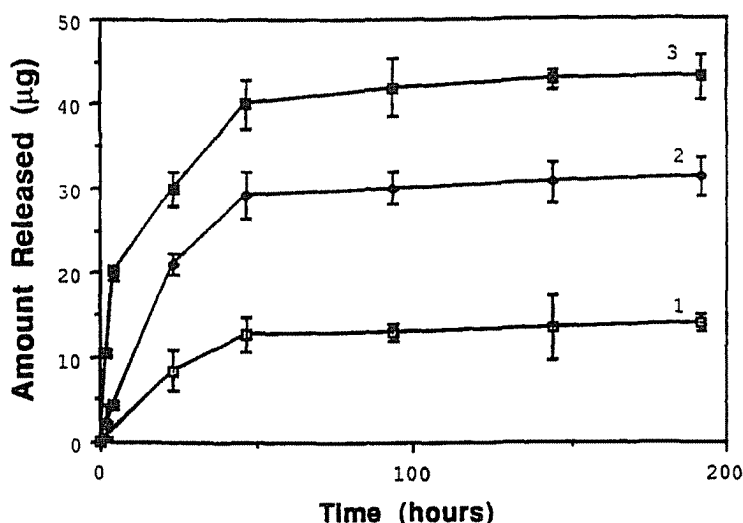

Fig. 7. Release of anti-*rev* mRNA from the following non-degradable hydrogels: 0.4KDA (curve 1); 6KDA (curve 2); and 10KDA (curve 3). The molecular weight of the PEG segment within the precursor was varied to examine the resultant changes in the hydrogel permeability.

examined independently of degradation. The higher the PEG molecular weight, the greater the permeability to the oligonucleotide, i.e., larger amounts of the oligonucleotide were able to diffuse out of the hydrogel matrix without the aid of degradation. However, only small amounts of the total entrapped oligonucleotide were able to diffuse out of the matrix.

4. Discussion

Hydrogels have been used extensively for controlled release applications, but most of these materials have been non-degradable polymers which release entrapped substances by diffusion and later require removal of the spent device. The photopolymerized hydrogel described here

is degradable, and as seen in Fig. 6, only a portion of the entrapped substance can be released by simple diffusion. Instead, release is regulated by degradation of the polymer. As the ester linkages in the oligomeric α-hydroxy acid segment are hydrolyzed, the pore sizes within the hydrogel matrix increase, and drug molecules are able to escape [3]. The fractional amount released by diffusion independently of degradation, depends on the molecular weight of the permeant and the molecular weight of the PEG chain in the hydrogel precursor. A small drug would require a low molecular weight PEG in the precursor to achieve release mediated by hydrogel degradation.

It is possible to control release rates of macromolecular drugs from these photopolymerized hydrogels through the design of the precursor. Release rates depend on both the permeability of the hydrogel prior to degradation and its degradation rate. Permeability depends on the crosslinking density, determined by the length of the PEG segment, and the degradation rate depends on both the length and composition of the α-hydroxy acid segment. Drug release from this type of hydrogel has been maintained for as long as 2 months in vitro [3]. Longer durations may be possible with precursors containing ϵ-caprolactone oligomers. Numerous variations on the acrylated PEG–co-α-hydroxy acid format can be synthesized, thus allowing one to essentially tailor the hydrogel to the requirements of a specific drug delivery application.

In the current study, in vitro release of proteins and oligonucleotides was evaluated to determine some of the parameters which must be understood before such tailoring for specific applications can be accomplished. Fig. 1 shows the release of tPA form a 10KL5 hydrogel, evaluated in terms of activity released. The initial release rate was slightly faster than release at later times. This period was correlated with the swelling of the hydrogel matrix to its equilibrium state, and it appears that the increased water flux during this time may be responsible for enhanced diffusion of the entrapped protein. A previous study [3] examined the release of proteins from hydro-

gels which were completely impermeable to the entrapped protein prior to degradation. In this case, the swelling effect was not observed, but instead there was a lag period with low release rates, and release increased at later times as the hydrogel degraded and became more permeable. It should also be noted that the photopolymerization process did not appear to adversely affect the activity of tPA, as all of the initial enzymatic activity was released from the gel.

Fig. 3 summarizes the release profiles of different molecular weight proteins, ranging from 6000 to 150,000 Da, from a 10KL5 hydrogel. A linear relationship was found between the molecular weight of the entrapped protein and the average release rate, as shown in Fig. 4, so it should be possible to predict the release rate of other proteins from the 10KL5 hydrogel. Similar plots will have to be constructed for other photopolymerized PEG hydrogels.

Fig. 5 shows the effect of slight variations in the degradation rate of the hydrogel, achieved by altering the length of the polylactidyl segment. Even the greatest difference in the amount of release, seen at day 5, was not statistically significant ($P > 0.2$). This suggests that the synthesis conditions do not need to be tightly controlled, as only large variations in the length of the α-hydroxy acid segment are able to significantly affect release rates. The composition of the α-hydroxy acid is probably a more effective means of altering the degradation rate.

As can be seen in Figs. 6 and 7, the hydrogel precursor must be matched to the molecular weight of the drug if drug release is to be controlled by the degradation of the hydrogel. For the 27mer antisense oligonucleotide to *rev*, approximately 11% was released passively (without degradation) from a hydrogel with a 10,000-Da PEG, 8% from a 6000-Da PEG, and only 3% from a 400-Da PEG. Thus, if the rapid initial release observed in Fig. 6 is to be reduced, the molecular weight of the PEG in the precursor must be reduced.

Perhaps a unique advantage of the photopolymerized PEG-based hydrogels described herein

over previously available materials is that they may be formed in situ. Hydrogels which are formed in contact with tissue adhere firmly to the underlying tissue [5], effectively localizing the hydrogel and the drug release. This feature may allow one to form a hydrogel upon a specific site, such as a tumor or the surface of a damaged blood vessel, and release the entrapped drug precisely where it is required. Localized drug release often allows one to use smaller doses and may help to minimize side effects of certain drugs. Future studies will compare the effect of localized release of protein drugs to systemic release in vivo using photopolymerized, biodegradable PEG hydrogels.

Acknowledgements

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Structure and properties of ovalbumin

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Abstract

Ovalbumin is a protein of unknown function found in large quantities in avian egg-white. Surprisingly, ovalbumin belongs to the serpin family although it lacks any protease inhibitory activity. We review here what is known about the amino acid sequence, post-translational modifications and tertiary structure of ovalbumin. The properties of ovalbumin are discussed in relation to their possible functional significance. These include reasons for failure of ovalbumin to undergo a typical serpin conformational change involving the reactive centre loop, which explains why ovalbumin is not a protease inhibitor, and also the natural conversion of ovalbumin to the more stable "*S*" form. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Food allergy; Ovalbumin

1. Introduction

Ovalbumin is the major protein in avian egg-white and was one of the first proteins to be isolated in a pure form [1]. Its ready availability in large quantities has led to its wide-spread use as a standard preparation in studies of the structure and properties of proteins, and in experimental models of allergy. In addition the synthesis of ovalbumin by hen oviduct and its regulation by steroid hormones has provided a model system in studies of protein synthesis and secretion. New interest in the structure and function of ovalbumin was stimulated by the unexpected finding that this protein belongs to the serpin super-family [2]. The serpins [3] are a family of more than 300 homologous proteins with diverse functions found in animals, plants, insects and viruses, but not in prokaryotes [4,5]. They include the major serine protease inhibitors of human plasma that control enzymes of the coagulation, fibrinolytic, complement and kinin cascades, as well as proteins without any known inhibitory properties such as hormone binding globulins, angiotensinogen and ovalbumin.

2. The serpins

The functional activity of serpins as protease inhibitors is dependent on their unique ability to undergo a dramatic conformational change (illustrated in Fig. 1) on interaction with an attacking protease. The native serpin (Fig. 1a) has a highly flexible peptide loop (yellow), known as the reactive centre loop, which holds the reactive centre and mimics a good proteolytic substrate. The reactive centre peptide bond that is attacked by the protease is denoted P1–P1′ using the nomenclature of Schechter and Berger [6] where P1 is N-terminal and P1′ is

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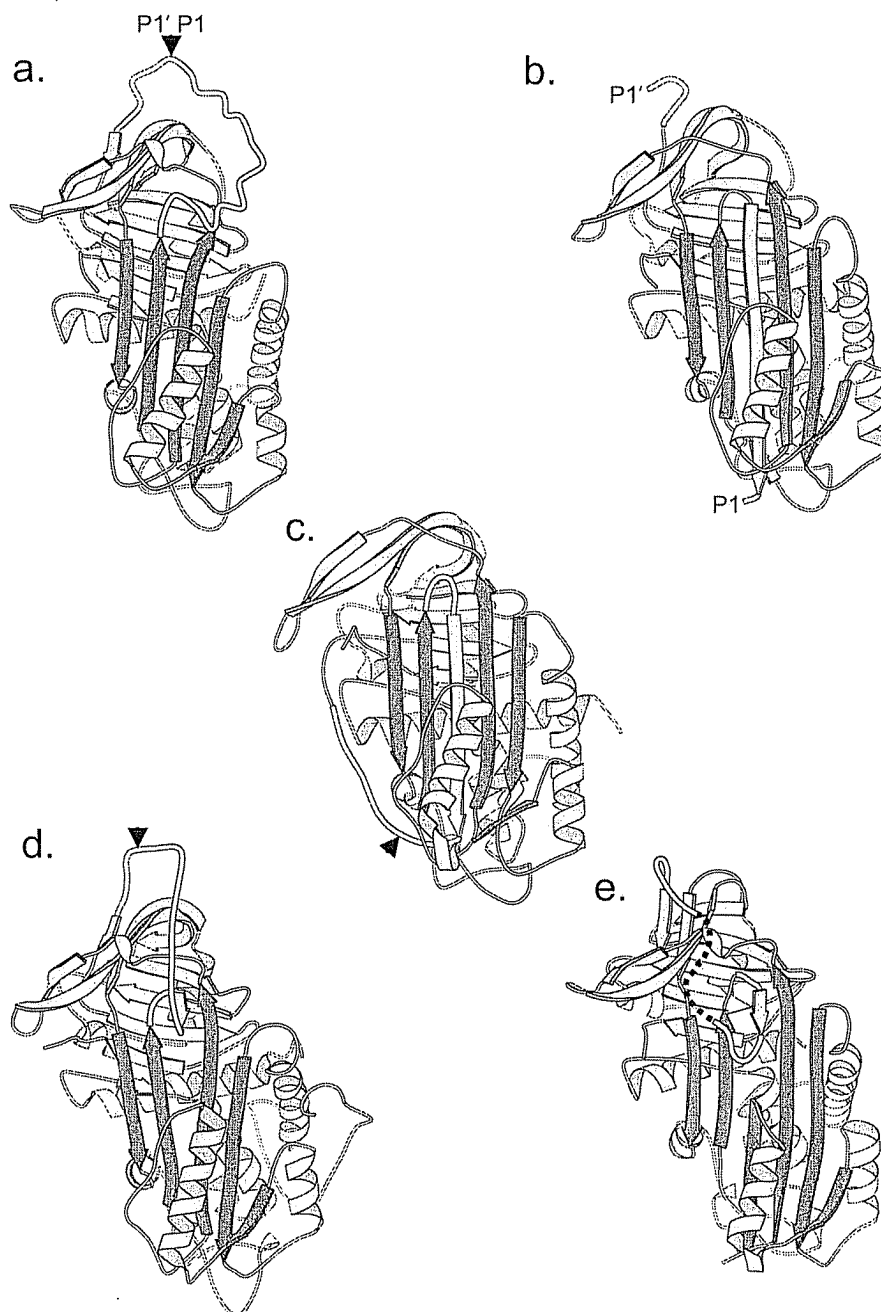*E-mail address:* penny@mrc-lmb.cam.ac.uk (P.E. Stein).

Fig. 1. The crystal structures of native α_1-antitrypsin, a, reactive centre cleaved α_1-antitrypsin, b, latent antithrombin, c, native antithrombin, d, and the delta variant of α_1-antichymotrypsin, e, illustrate the conformational mobility of the serpin superfamily. Inhibitory serpins react with proteases via an exposed, flexible reactive centre loop (yellow) which mimics a good substrate and leads to cleavage of the reactive centre bond, P1–P1', indicated by an arrow. Cleavage results in subsequent incorporation of the N-terminal segment of the reactive centre loop as the fourth strand of the main β-sheet A (red). If deacylation occurs before full incorporation of the reactive centre loop the serpin is a proteolytic substrate, but if loop insertion is rapid, the protease becomes trapped while still attached to P1 via an ester bond. Loop insertion in the absence of cleavage also occurs. Latent antithrombin, c, is fully loop inserted and completely inactive. Antithrombin and plasminogen activator inhibitor-1 have been shown to convert to this conformation naturally in vivo. The partial insertion of the native antithrombin reactive centre loop, d, accounts for its need for the cofactor heparin to achieve rates of protease inhibition typical of other serpins. Serpins are sensitive to mutations which alter their conformational state. A recent structure of a pathogenic variant of α_1-antichymotrypsin, e, reveals another partially loop-inserted conformation resulting in complete loss of activity.

C-terminal to the site of cleavage. Unlike most proteins, the native serpin fold is not the most thermodynamically favoured conformation but a metastable folding intermediate. Interaction with the target protease releases conformational constraints and results in incorporation of the reactive centre loop as the middle strand (strand 4A) in the central β-sheet A (red) (Fig. 1b). The loop inserted form is more than twice as stable as the native conformer. It has recently been shown that full insertion of the reactive centre loop in β-sheet A, as illustrated in Fig. 1b, is required for inactivation of the protease [7–9], and that the energy of the conformational change is used to destabilise and distort the protease while still attached to the serpin via an ester bond at P1 [10]. The ability of serpins to undergo such a conformational change allows for tight regulation of their activity and provides a mechanism for clearance of serpin–protease complexes. However the dependence of the inhibitory mechanism on this complex structural transition makes the serpins especially sensitive to mutations which cause loss of function either by hindering loop insertion or by causing loop insertion in the absence of proteolytic attack (premature loop insertion) [11,12]. Premature loop insertion may occur within a single molecule (intramolecular loop insertion) or between molecules (intermolecular loop insertion). Intermolecular loop insertion, resulting in serpin polymerisation, is associated with various disease states including liver disease [13] and dementia [14].

The complex control mechanisms seen in inhibitory serpins explain the evolutionary success of this family as the predominant protease inhibitors in higher organisms. But what about the serpins without inhibitory functions? Many non-inhibitory serpins have simple roles: the hormone binding globulins act as ligand carriers in the circulation [15,16]; the only known function of angiotensinogen lies in its amino terminus which is cleaved by renin to release a small peptide involved in the control of blood pressure [17]. The function of ovalbumin is still unknown. It is interesting to consider why such a large and complex molecule as a serpin has been utilised for such apparently simple tasks and it is tempting to speculate that the serpin framework may have regulatory functions yet to be discovered.

In serpins without inhibitory activity a putative reactive centre can usually be readily identified by sequence alignment with typical inhibitory serpins. In ovalbumin, this is Ala–Ser at residues 353–354 which is also the sole cleavage site for elastase as predicted by the sequence [18]. Intriguingly, some non-inhibitory serpins have apparently retained the mobility of the reactive centre loop typical of inhibitory members of the family. The hormone binding globulins, corticosteroid binding globulin and thyroxine binding globulin, both undergo the characteristic serpin conformational change following cleavage at their putative reactive centre by enzymes released from activated neutrophils [19]. In corticosteroid binding globulin, the conformational change is associated with a reduction in hormone binding affinity suggesting a possible physiological mechanism for release of cortisol at inflammatory sites [19]. Other non-inhibitory serpins, including angiotensinogen and ovalbumin, do not show evidence for a large conformational change following cleavage at their putative reactive centres [20,21] and appear to have lost the extreme mobility of their inhibitory ancestors.

3. Amino acid sequence

Ovalbumin is a glycoprotein with a relative molecular mass of 45 000. The amino acid sequence of hen egg-white ovalbumin comprising 386 amino acids was deduced from the mRNA sequence by McReynolds et al. [22] and is in agreement with sequences of the purified protein [23] and the cloned DNA [24]. The sequence includes six cysteines with a single disulfide bond between Cys74 and Cys121 [25]. The amino terminus of the protein is acetylated [26]. Ovalbumin does not have a classical N-terminal leader sequence, although it is a secretory protein. Instead, the hydrophobic sequence between residues 21 and 47 may act as an internal signal sequence involved in transmembrane location [27]. Two genetic polymorphisms of ovalbumin have been reported: a Glu→Gln substitution at residue 290 [28] and an Asn→Asp substitution at residue 312 [29]. The sequences of chicken, Japanese quail, and common turkey ovalbumins have been determined and are 90% identical. Major differences include: a truncation of three amino acids from P1–P2′ in quail

ovalbumin, which supports the theory that ovalbumin is not inhibitory; an extra glycosylation site at 372 in turkey ovalbumin; chicken ovalbumin has six Cys residues while quail and turkey ovalbumin lack Cys31. As more ovalbumin sequences become available it may be possible to determine which domains are required for function through sequence comparison.

4. Post-translational modifications

A single carbohydrate side chain is covalently linked to the amide nitrogen of Asn293 at a typical Asn–X–Thr sequence recognised by glycosyltransferases. A second potential recognition site, Asn–X–Ser at residues 317–319, is not glycosylated in the secreted form found in the egg white, but has been observed transiently in the oviduct [30]. The carbohydrate chain is heterogeneous but the different ovalbumin glycopeptides share a common core structure: mannose β(1–4) glcNAc β(1–4) glcNAc–Asn293 [31]. Ovalbumin has two potential phosphorylation sites at serines 69 and 345. Comparison of ovalbumin sequences in different avian species shows an invariant glutamic acid two residues C-terminal to each phosphoserine which may form part of a recognition site for a protein kinase. Heterogeneity in the electrophoretic behaviour of ovalbumin is largely due to different degrees of phosphorylation at these sites [23,32]. Three major fractions can be separated by ion-exchange chromatography with, respectively two, one and zero phosphate groups per ovalbumin molecule in an approximate ratio of 8:2:1 [33,34].

5. Tertiary structure

The first serpin structure to be solved was of human α_1-antitrypsin that had been proteolytically cleaved at its reactive centre peptide bond. It showed an unexpected separation of the new chain termini by 70 Å (Fig. 1b) [35]. The N-terminal portion of the cleaved reactive centre loop forms the fourth strand of the six stranded β-sheet A that runs parallel to the long axis of the molecule. It was predicted that in intact antitrypsin this strand would be withdrawn

from sheet A to form an external peptide loop, and β-sheet A would have only five strands. The first crystallographic models for the native serpin structure came from structures of ovalbumin (Fig. 2). The structure was initially solved for a proteolytically modified form of ovalbumin called plakalbumin [33]. Plakalbumin is formed by the interaction of ovalbumin with subtilisin, which excises six amino acids from the reactive centre loop [18]. The crystal structure of plakalbumin was reported by Wright et al. [36] and was refined at a resolution of 2.8 Å. The cleaved ends are separated by 27 Å in this structure, but the conformational change seen in cleaved inhibitory serpins, in which the cleaved reactive centre loop is inserted in β-sheet A as a new strand, has not taken place (Fig. 2b). In plakalbumin, sheet A has only five strands as had been predicted for an intact inhibitory serpin, but the plakalbumin structure provided only a partial model for an intact serpin since the reactive centre loop had been cleaved [37]. The conformation of the intact reactive centre loop was still unknown.

The crystal structure of native hen ovalbumin [38,39] was the first structure of an uncleaved serpin, and surprisingly, the intact reactive centre loop was found to take the form of an exposed α-helix of three turns that protruded from the main body of the molecule on two peptide stalks (Fig. 2a). The overall structural similarity between ovalbumin and active inhibitors of the serpin family suggested that the ability to adopt a helical form might be common to all serpins, although it would be an unexpected feature of a protease inhibitor since a helical reactive centre would have to unfold to dock to a protease. The ovalbumin structure includes four crystallographically independent ovalbumin molecules and the position of the helical reactive centre loop relative to the protein core differs by 2–3 Å between molecules. Although this shift is probably due to the different environments of the helices in the crystal lattice, it suggested that the reactive centre loop is flexible in solution.

The structures of various uncleaved inhibitory serpins have now been determined and confirm the exceptional mobility of the serpin reactive centre loop. These include inactive conformations resulting from intramolecular loop insertion in plasminogen activator inhibitor-1 [40] and in antithrombin [41]
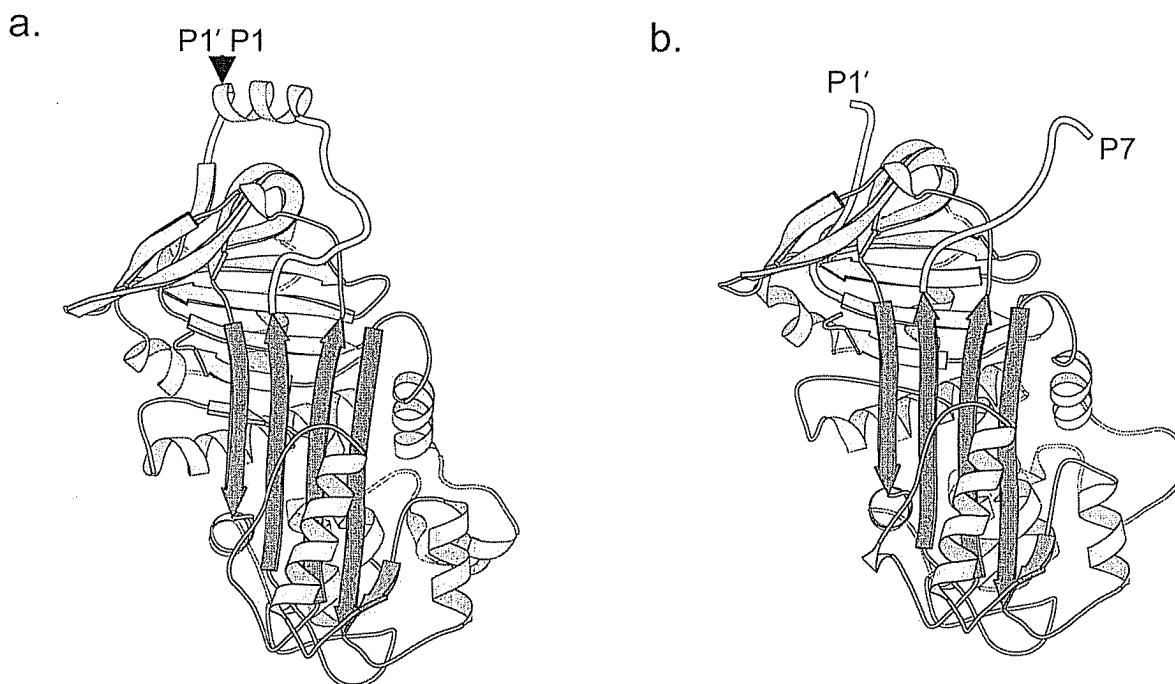
a.

P1′ P1

b.

P1′

P7

Fig. 2. The crystal structures of native, a, and subtilisin cleaved, b, ovalbumins. Native ovalbumin possesses a typical serpin fold, with a three-turn α-helical reactive centre loop (yellow). Unlike the inhibitory serpins, cleavage within the reactive centre loop does not result in its incorporation into β-sheet A (red). Subtilisin cleavage to form "plakalbumin" results in the excision of P1 to P6 and no loop insertion.

(Fig. 1c). Several serpins have now been crystallised in active conformations including a form of α_1-antichymotrypsin with a distorted helical reactive centre loop [42], an extended loop conformation in active plasminogen activator inhibitor-1 [71] and partially loop-inserted conformations of native antithrombin [41,43] (Fig. 1d) and a variant of α_1-antichymotrypsin [44] (Fig. 1e). In some of these crystal structures, the conformation of the reactive centre loop appears to be significantly influenced by packing contacts. However, the recent crystal structures of α_1-antitrypsin [45] and Manduca sexta serpin 1K [46] have shown that the serpin reactive centre loop can adopt a canonical conformation that would readily dock with a target protease. Structural studies of serpins in various conformations have shown how their unique flexibility is essential for function, and have formed the basis for a detailed understanding of the normal and abnormal function of this unusual family of proteins at a molecular level [11].

6. Ovalbumin is not a protease inhibitor

A comparison of the structures of native ovalbumin (Fig. 2a) and plakalbumin (Fig. 2b) shows that cleavage within the reactive centre loop of ovalbumin does not result in its incorporation into β-sheet A [20,21,36]. This failure of full loop insertion, which is an essential requirement for protease inhibition by serpins, explains why ovalbumin shows no inhibitory activity despite its sequence homology with functional inhibitors of the serpin family. But what is the molecular explanation for the failure of ovalbumin to undergo this conformational change?

In an active inhibitory serpin, it is generally accepted that insertion of the reactive centre loop into β-sheet A takes place sequentially, starting with P15, and in register with that observed in the structures of cleaved serpins. Such incorporation of a loop into a β-sheet results in alternating side chains being buried (Fig. 3). In typical inhibitory serpins
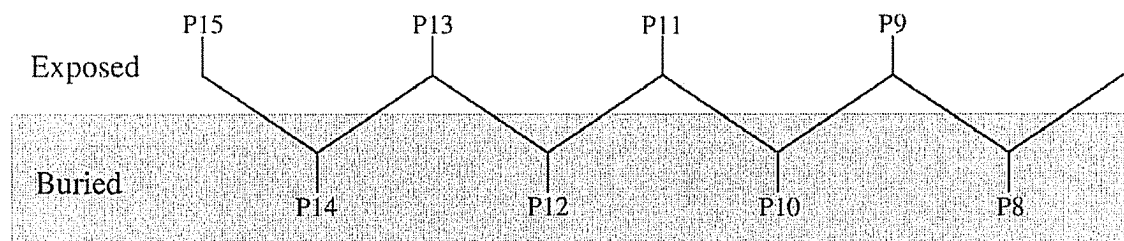
Fig. 3. Insertion of the serpin reactive centre loop into β-sheet A results in every other side chain being buried in the hydrophobic core of the serpin. The portion of the reactive centre loop most distant from the reactive centre bond (P15–P8) is called the hinge region and is crucial for efficient loop insertion. Each even numbered residue is buried and thus the sequence of this region is highly conserved as small amphipathic or hydrophobic amino acids.

the even numbered P residues of the reactive centre loop from P14 to P4 would have their side chains buried and are commonly small amphipathic or hydrophobic amino acids (Table 1). The first side chain to insert, P14, is thus critical for continued sequential incorporation of the reactive centre loop and is highly conserved as threonine among the inhibitory serpins. The arginine at the P14 position in ovalbumin led to the hypothesis that ovalbumin is thermodynamically incapable of loop insertion since burying the bulky charged side chain would be energetically too costly [36]. In ovalbumin, other residues in the so-called "hinge region" from P16 to P8 deviate from the conserved sequence (Table 1), notably P12 to P10 is VVG in ovalbumin compared with AAA in inhibitory serpins. The sequences in the hinge region of other non-inhibitory serpins are shown in Table 1, and in all cases there is a residue with an even P number which differs significantly from the conserved sequence and is often charged or bulky.

Work on recombinant variants of ovalbumin con-

firmed the hypothesis that bulky residues in the hinge region prevented loop insertion upon cleavage of the reactive centre loop [47]. Loop insertion is accompanied by a large increase in the thermal stability of serpins and is thought to be the driving force for the translocation and inactivation of proteases [10,48]. Whereas a cleaved inhibitory serpin is at least 60°C more stable than its native counterpart, cleaved ovalbumin is 1 to 2°C less stable than the native form [47,49]. An increase in stability on cleavage can be used as a marker for serpin reactive centre loop insertion. Variant ovalbumin with P14 arginine replaced by serine does undergo loop insertion on cleavage as indicated by an 11°C increase in thermal stability, and a further 5°C increase in stability is observed for the cleaved variant which includes the consensus P12 to P10 AAA [47]. Although these experiments demonstrate that the hinge region sequence of ovalbumin is responsible for its apparent lack of ability to spontaneously loop insert upon cleavage, it does not fully explain why ovalbumin is not inhibitory. An inverse mutation was made in the

Table 1
Comparison of the hinge region residues for non-inhibitory serpins with the consensus inhibitory sequence

	P16	P15	P14	P13	P12	P11	P10	P9	P8	Ref.
Inhibitory consensus	Glu	Gly	Thr	Glu	Ala	Ala	Ala	Ala	Thr	[4]
% Identity	38	98	80	85	98	75	65	75	83	[4]
Non-inhibitory serpins										
Ovalbumin	Ala	Gly	Arg*	Glu	Val*	Val	Gly	Ser	Ala	[22]
Human angiotensinogen	Asp	Glu	Arg*	Glu	Pro*	Thr	Glu*	Ser	Thr	[68]
Maspin	Glu	Ile	Thr	Glu	Asp*	Gly	Gly	Asp	Ser	[69]
PEDF	Asp	Gly	Ala	Gly	Thr	Thr	Pro*	Ser	Pro*	[70]

Residues which have an asterisk are considered detrimental to loop insertion.

inhibitory serpin antitrypsin where the consensus P14 threonine was replaced by arginine [50]. This variant was still capable of loop insertion and showed an increase in thermal stability comparable to wild-type. Furthermore, it retained the ability to inhibit certain target proteases, although with significantly diminished efficacy. Another reason to suspect that other factors may contribute to the lack of inhibitory activity of ovalbumin is the relatively small increase in thermal stability of the cleaved ovalbumin variants. The increase in denaturation temperature from 11 to 16°C for the cleaved combined variant with the P14, P12–P10 mutations indicates that insertion involves at least P14 through P12. Proteolytic susceptibility of the remainder of the reactive centre loop after cleavage at P1–P1′ indicate that the P8 residue is accessible to proteases and therefore not incorporated into β-sheet A. Thus, although the hinge region residues of ovalbumin are apparently incompatible with incorporation into β-sheet A upon cleavage, this provides only part of the reason for the lack of inhibitory properties of ovalbumin, since even with consensus hinge region residues ovalbumin is not capable of the full reactive centre loop incorporation observed for cleaved inhibitory serpins and required for protease inhibition. Support for this hypothesis is provided by work on a chimera of ovalbumin which required 64% of the sequence of PAI-2 before any inhibitory properties were observed [51].

7. *S*-Ovalbumin

By the time eggs reach the supermarket shelf and ultimately the consumer, typically more than half of the ovalbumin has changed form. *S*-Ovalbumin was discovered in 1964 by following the change in the melting profile of ovalbumin with the age of eggs [52]. The mid-point of thermal denaturation (Tm) of ovalbumin shifted from 78 to 86°C, and the new form was named "*S*-ovalbumin" to denote its increased stability [53]. The appearance of *S*-ovalbumin coincides with the loss of the "food value" of eggs since eggs with high *S*-ovalbumin content have runny whites and do not congeal as effectively on cooking. Most of the work on *S*-ovalbumin has been motivated by the loss in food value of stored eggs

and not in relation to the potential function of ovalbumin in eggs.

S-Ovalbumin is easily formed in vitro by a 20 h incubation at 55°C in 100 m*M* sodium phosphate, pH 10 [54]. The high pH and temperature increase the rate of conversion, the basis of which has been extensively studied. Biochemical studies comparing the properties of native and *S*-ovalbumin confirm that the increase in stability on conversion to the *S*-form results from a unimolecular conformational change and not a change in the chemical make-up of ovalbumin [53,55,56]. The *S*-form differs from native ovalbumin only in its greater stability, compactness and hydrophobicity [57]. The conformational conversion requires the disulfide bond between cysteines 74 and 121 [58]. The chemically denatured states of native and *S*-ovalbumin also differ so that renaturation of *S*-ovalbumin does not lead to the native conformer [59]. *S*-Ovalbumin has also been formed from recombinant ovalbumin produced in *Escherichia coli* providing unequivocal evidence that the conversion is not dependent on post-translational modifications [60].

Only recently has the conversion of ovalbumin to the *S*-form been addressed in light of its membership in the serpin superfamily [61]. Most inhibitory serpins are capable of intramolecular reactive centre loop insertion in the absence of cleavage to form a "locked" or "latent" conformation [62]. Structures of such conformations have recently been determined by X-ray crystallography, including latent plasminogen activator inhibitor-1 and antithrombin (Fig. 1c), native antithrombin (Fig. 1d), and the "delta" conformer of α_1-antichymotrypsin (Fig. 1e). Each of these loop-inserted forms are more stable than the native (non-loop inserted) five-stranded β-sheet A forms. Recent work based on biochemical and thermal stability studies, has provided evidence that the conformational change in *S*-ovalbumin is also an intramolecular insertion of the reactive centre loop [61]. Studies using circular dichroism and Fourier transform infrared spectroscopies concluded that the limited conformational change in *S*-ovalbumin involves a small 2–5% loss of α-helix content and a concomitant increase in anti-parallel β-sheet [61,63]. This is what would be expected if the α-helical reactive centre loop of ovalbumin were to unravel to allow for limited incorporation into β-sheet A.

Consistent with an unravelled helix are the observations that the reactive centre loop of S-ovalbumin shows increased proteolytic susceptibility and loss of contacts with the main body of the molecule involving glutamates at positions P13 and P7 [61,64].

A working model for the conformation of S-ovalbumin was proposed to be a locked conformation with partial loop insertion from P15 to P10 [61]. This limited extent of loop insertion is necessary to explain the unaltered rate of dephosphorylation of the phosphoserine at P9 in S-ovalbumin. The model includes insertion of the P14 arginine to preserve the register of reactive centre loop insertion observed for the cleaved inhibitory serpins. However, the recent crystal structure of the cleaved P14 arginine variant of α_1-antichymotrypsin shows that the P14 arginine side-chain is not buried, but in-register insertion occurs C-terminal to P14 [65]. Out-of-register β-sheet A incorporation of serpin reactive centre loops and related peptides has been demonstrated structurally [66]. This allows for the postulation of out-of-register incorporation of the reactive centre loop of ovalbumin as the basis of conversion to S-ovalbumin to avoid insertion of the P14 arginine side-chain. The model is limited by the constraints that only P15 to P10 can be involved and that no charged side-chains may be buried. The improvement in stability observed for the cleaved P14, P12–P10 variant compared to the P14 variant of ovalbumin allows for the minimum number of residues which can be accommodated as strand 4 in β-sheet A to be set at six (P15–P12). The structure of S-ovalbumin is thus likely to be partially loop inserted in a manner analogous to other members of the serpin superfamily (Fig. 1d and e), and may involve out-of-register loop insertion to avoid burying the P14 arginine side chain.

8. Conclusion

Although ovalbumin comprises 60–65% of the total protein in egg white, its function remains unknown. Ovalbumin shows no protease inhibitory activity despite sequence identity of about 30% with antitrypsin and other functional inhibitors of the serpin family. The Ala–Ser bond at its putative reactive centre suggests specificity for elastase, but ovalbumin acts as a substrate not as an inhibitor of this enzyme. We believe that the structure of S-ovalbumin may be a central clue to determining the function of ovalbumin in eggs. The preservation of the serpin fold and its metastability is presumably not accidental and the natural conversion of ovalbumin to the more stable S-form is likely to be functionally relevant. The fraction of native ovalbumin may range from 20 to 80% in commercial preparations, and from 20 to 40% in fresh preparations from old eggs [53]. Perhaps the activity of ovalbumin has been overlooked due to inadvertent contamination with the S-form. It is possible that the activity of ovalbumin is lost on its conversion to S-ovalbumin, and thus the slow natural conversion provides a timing mechanism by which the needs of a chick embryo at different developmental stages can be met by the same protein. An alternative possibility is that the active form is the more stable S-form. This has recently been suggested by the finding of rapid conversion and subsequent migration to the chick embryo of a more stable form of ovalbumin found in fertilised eggs [67]. This more stable form has properties indistinguishable from that of S-ovalbumin formed in vitro, with the exception of an apparent loss of a phosphate group. It is thus possible that ovalbumin plays a role in chick embryo development which is mediated by the natural metastability of ovalbumin and affected through binding to an S-ovalbumin receptor in a phosphorylation dependent manner.

Acknowledgements

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